Figure 1. Typical, prior art, reaction scheme.

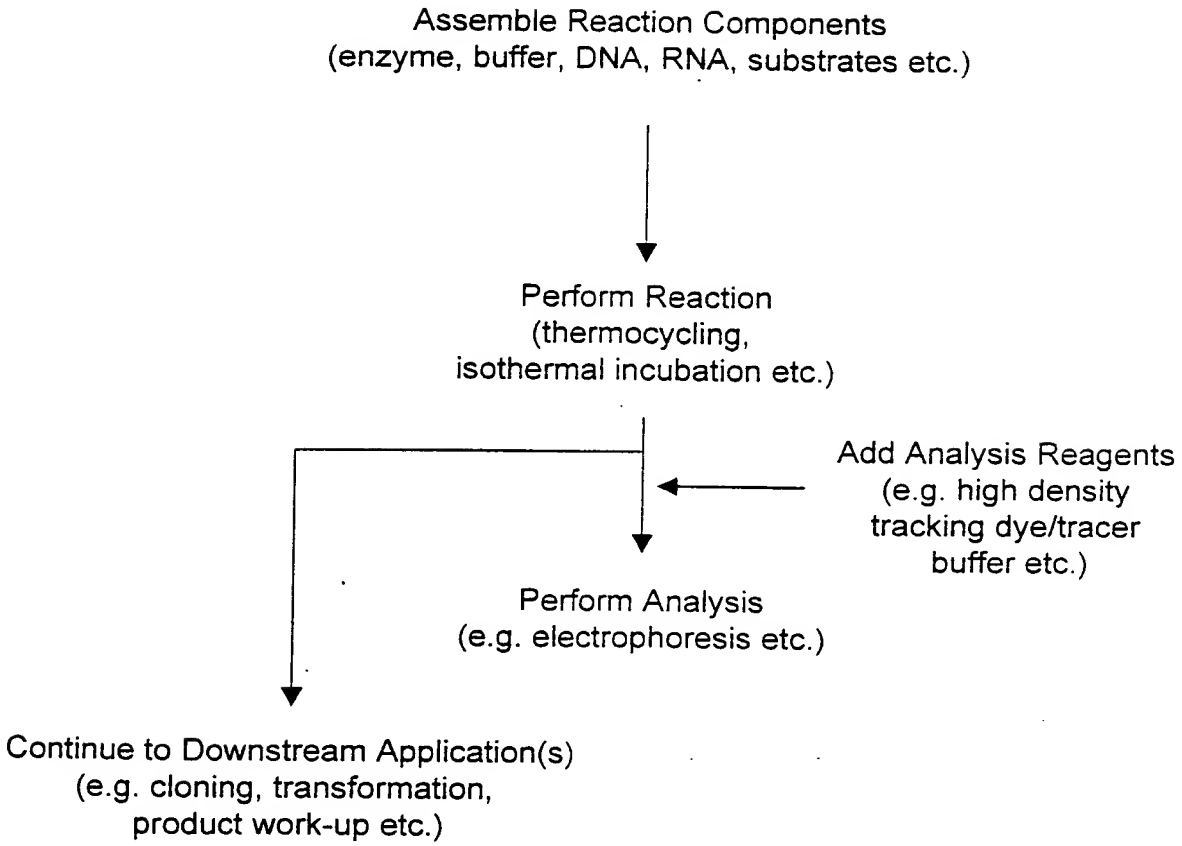

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Figure 2. Modified, prior art, reaction scheme

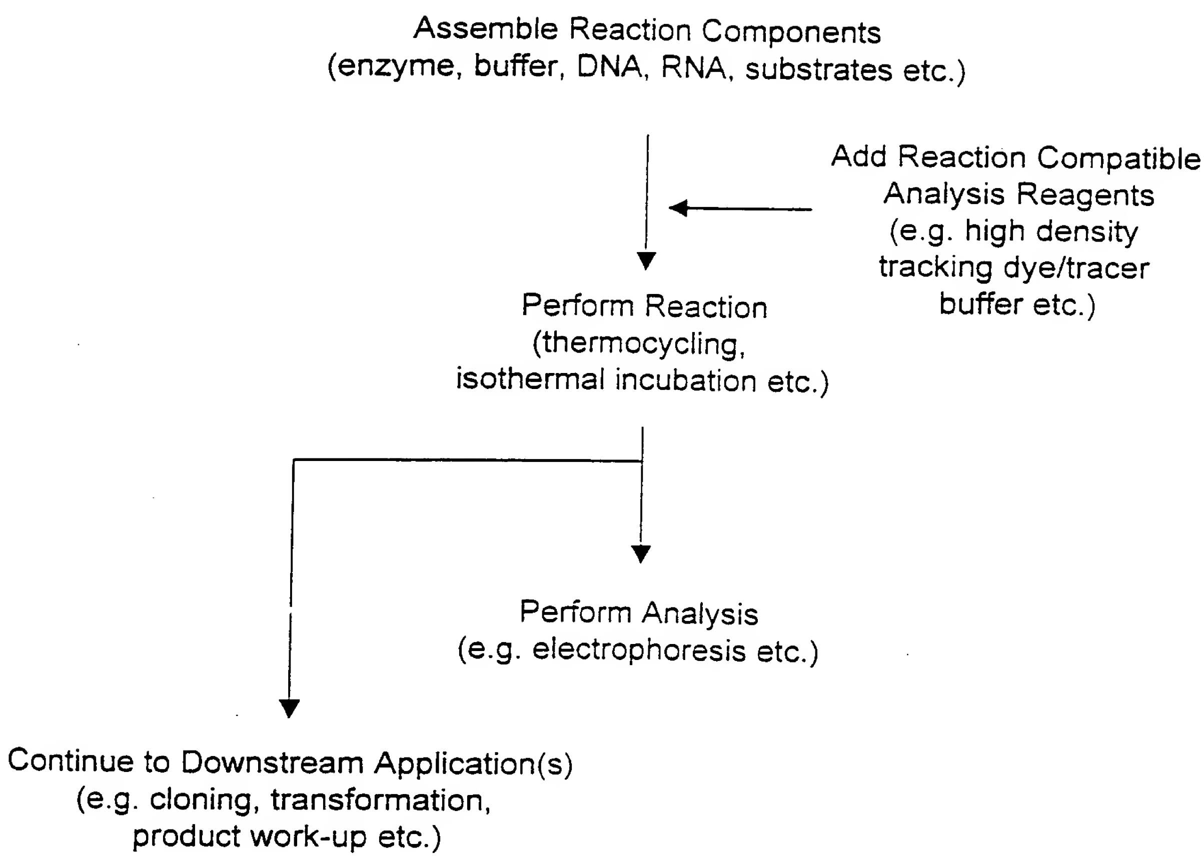

Figure 3. New reaction scheme using present invention. Any or all reaction components could be formulated to contain analysis reagents.

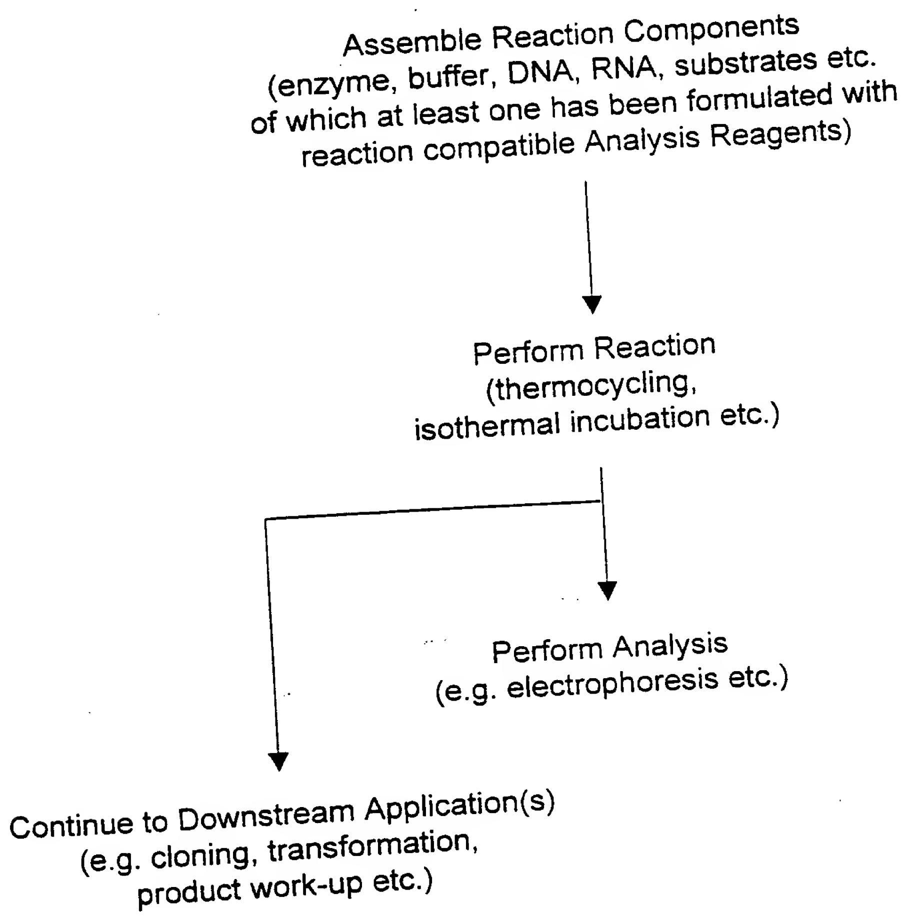

Figure 4. Reaction compatible analysis reagent identification and optimization for PCR, sequencing, restriction digestions etc.

Physical characteristics (e.g. high density to enable gel loading, colored to visualize gel loading, anionic chromophore to track electrophoresis progress, color (if desired)).

↓

Assemble collection of reagents for screening (e.g. collect a large sampling of anionic dyes).

↓

Screen molecules for compatibility. The screening might be best carried out by prioritizing the desired properties from least to most laborious (e.g see red Taq Figure 5).

↓

Formulate reagent, characterize reaction products (qualitative and quantitative). Optimize other reaction components for perturbation (if necessary). Final characterization (reaction product quality and quantity) and limitations (e.g. incompatible with specific downstream applications)

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Figure 5. Red Taq development.

Physical characteristics.
High density to enable gel loading- formulate enzyme dilute enough so that enough glycerol will be contained in a 2.5 unit per 50 microliter reaction.
Colored to visualize gel loading and act as a tracking dye- red anionic water and ethanol soluble dyes were sought.

↓

Assemble collection of reagents for screening - 40 anionic "red" (lambda max =450-550) dyes were selected as candidates.

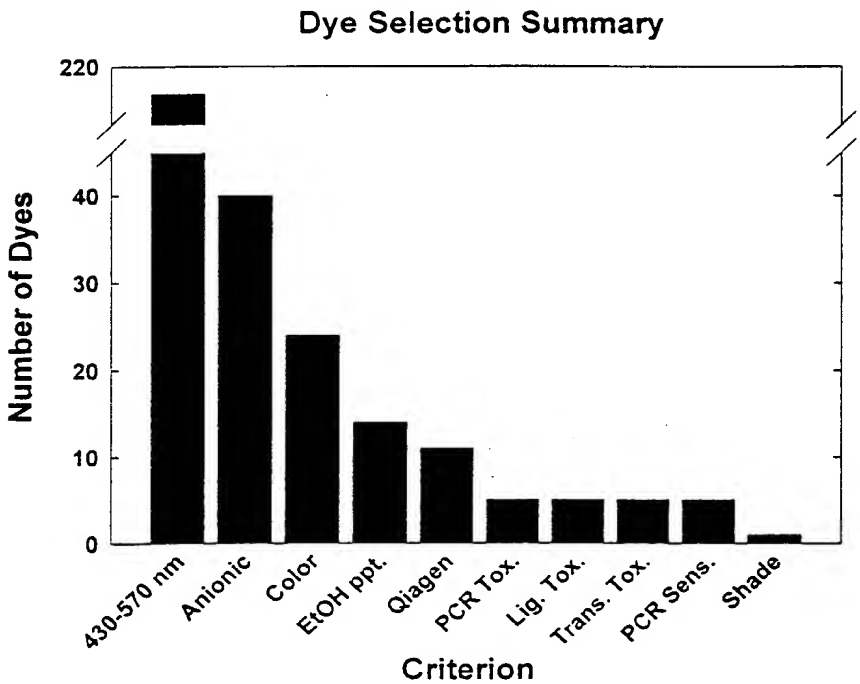
↓

Screen molecules for compatibility-summarized in Figure 6 . The dyes were scrutinized in the order:
1. Color (too yellow or purple thrown out)
2. Ethanol precipitation (colored DNA pellets thrown out)
3. Chaotropic salt/silica DNA purification (colored product thrown out)
4. PCR toxicity (Figure 7, low or no yield thrown out).
5. Ligase toxicity (Figure 8, low or no yield thrown out).
6. Transformation toxicity (Figure 9, low or no yield thrown out).
7. Remaining dyes more or less equivalent, submit to marketing for color selection.

↓

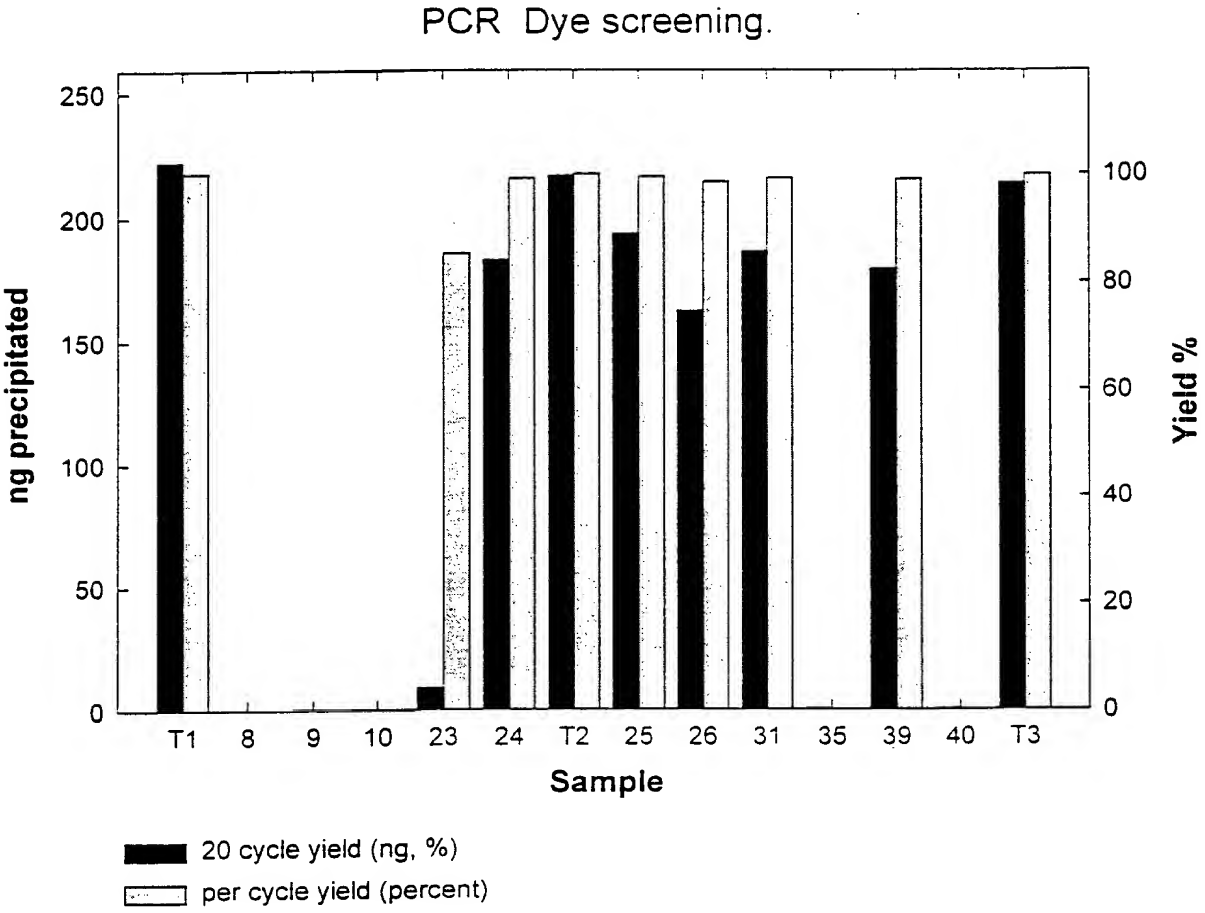
Formulate reagent- 80% acid red 1, 20% acid violet 5 (100%= absorbance of acid red 1 at lambda max + absorbance of acid violet 5 at lambda max.) to absorbance total =300 in Taq DNA polymerase at 1 unit per microliter in Taq storage buffer (20 mM Tris- HCl, pH 8.0, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% Tween 20, 0.5% Igepal CA- 630, 50% glycerol)
Characterize reaction products-product yields low relative to absence of dye reactions (Figure 10). Dye purity (Figure 11) and counter ion identity (Figure 12a,b) investigated for PCR toxicity/compatibility. Purified Mg acid red 1 and Mg Acid violet 5 found to be satisfactory.
Optimize other reaction components- Mg dyes contribute approximately 0.4 mM "free" Mg to PCR (Figure 13), 10X PCR buffer adjusted from 15 to 11 mM to accommodate Mg dye contribution. **Final characterization-** quality: gel (Figure 14), quantity (Figure 15). Limitations- will test with a panel of restriction enzymes, does not impact fluorescent sequencing.

Figure 6. Summary of PCR friendly dyes.



430-570 nm- visible absoprtion max.
Anionic- anionic dyes
Color-not too yellow/orange or purple
EtOH ppt.- did not co-precipitate with DNA
Chaotropic salt/silica purification (Qiagen PCR columns)- isolated DNA was colorless
PCR Tox.-little impact upon ^{32}P PCR product yield
Lig. Tox.- little to no effect upon ligation of lambda *PstI* fragments
Trans. Tox.- no effect upon ligation/transformation of *Eco*RI-pUC19
PCR Sens.- amplification similar to no dye as a function of template concentration.
shade- marketing

Figure 7. Relative toxicity of various dye candidates. Yields are derived from TCA insoluble counts of ^{32}P containing PCR reactions.



T1, T2 and T3= Taq controls (no dye) as in block and precipitation, numerals are dye number.
per cycle yield calculated assuming $y_{20}=y_1^{20}$ where y_{20} is the 20 cycle yield (measured) and y_1 is the per cycle yield.

Dye	y_{20} (%)	y_1(%)
23	9.04	85.3
24	84.1	99.1
25	88.9	99.4
26	74.6	98.5
31	85.6	99.3
39	82.4	99.0

Figure 8. Relative toxicity of various dye candidates on ligation. DNA is PstI digested lambda at 0.5 micrograms per microliter in 1X ligase buffer and 1mM ATP. Reactions were at 16°C for 1 hour. t_0 is without ligase, CTL is +ligase and - dye, 24,25,26,31,39 are dye candidates at concentrations suitable for gel loading and tracking +ligase.

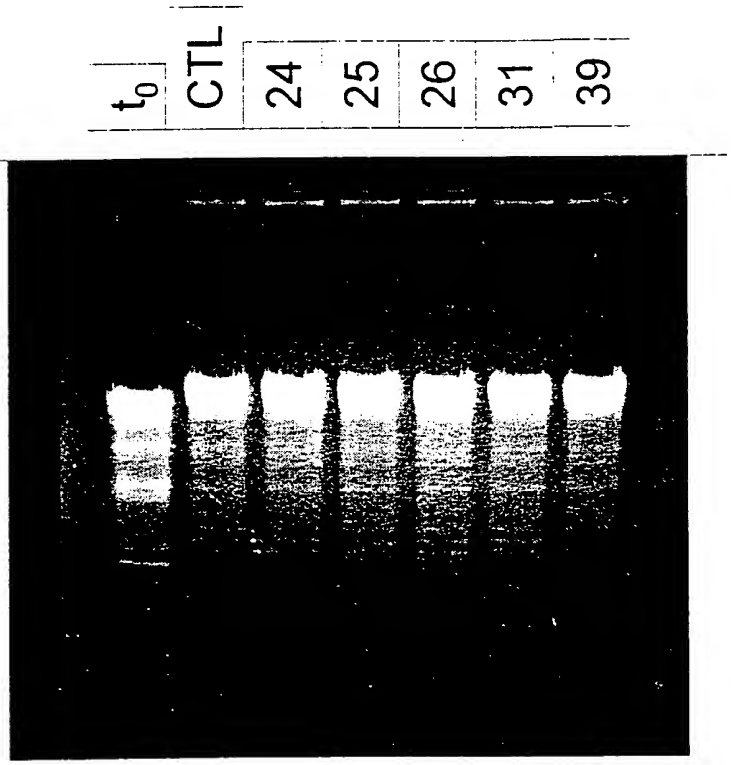


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Figure 9. Relative toxicity of various dye candidates on ligation followed by transformation. DNA is HindIII cut pUC19 that was ligated in presence and absence of various dyes. Ligations were as described in Figure 8. Transformation conditions were as described for DH5α competent cells (Life Technologies. -lig is without ligase, +lig is +ligase and - dye, 24,25,26,31,39 are dye candidates at concentrations suitable for gel loading and tracking +ligase.

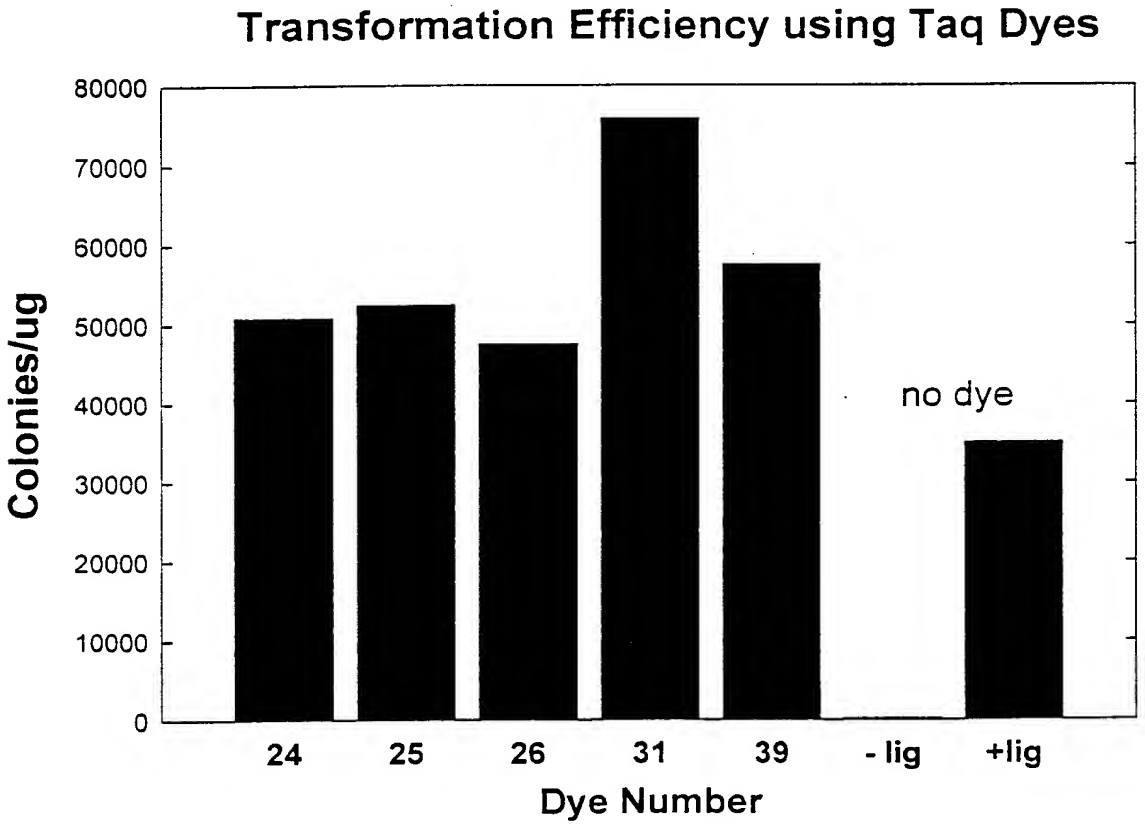

Figure 10. Relative PCR yields using red (acid violet 5) and white Taq DNA polymerase. Target=lambda 500mer (Perkin-Elmer control), 25 cycles (94,55,72°C at 1 min each), [Taq]=0.05 units per microliter, replicate=4. Yield from TCA precipitable counts (^{32}P).

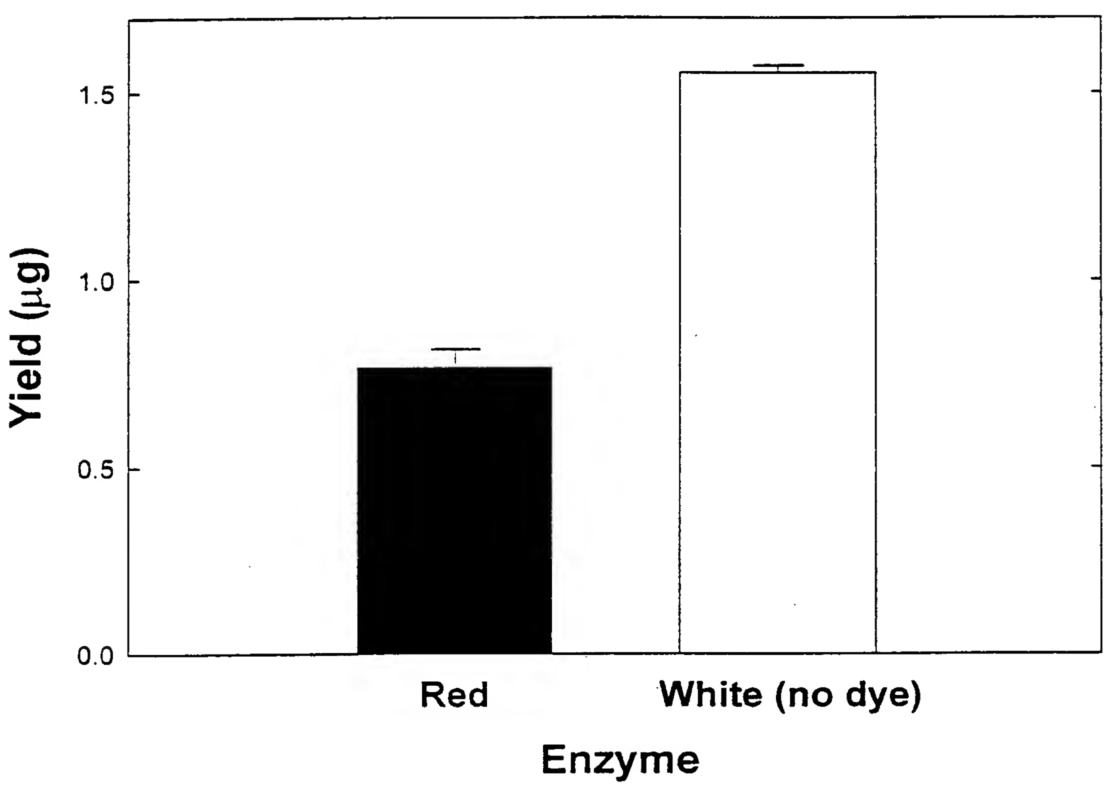

Figure 11. PCR yield of crude vs. pure (desalted, C18 resin, sep-pak, Millipore) acid violet 5 containing reactions. Target and PCR conditions were as described in Figure 10.

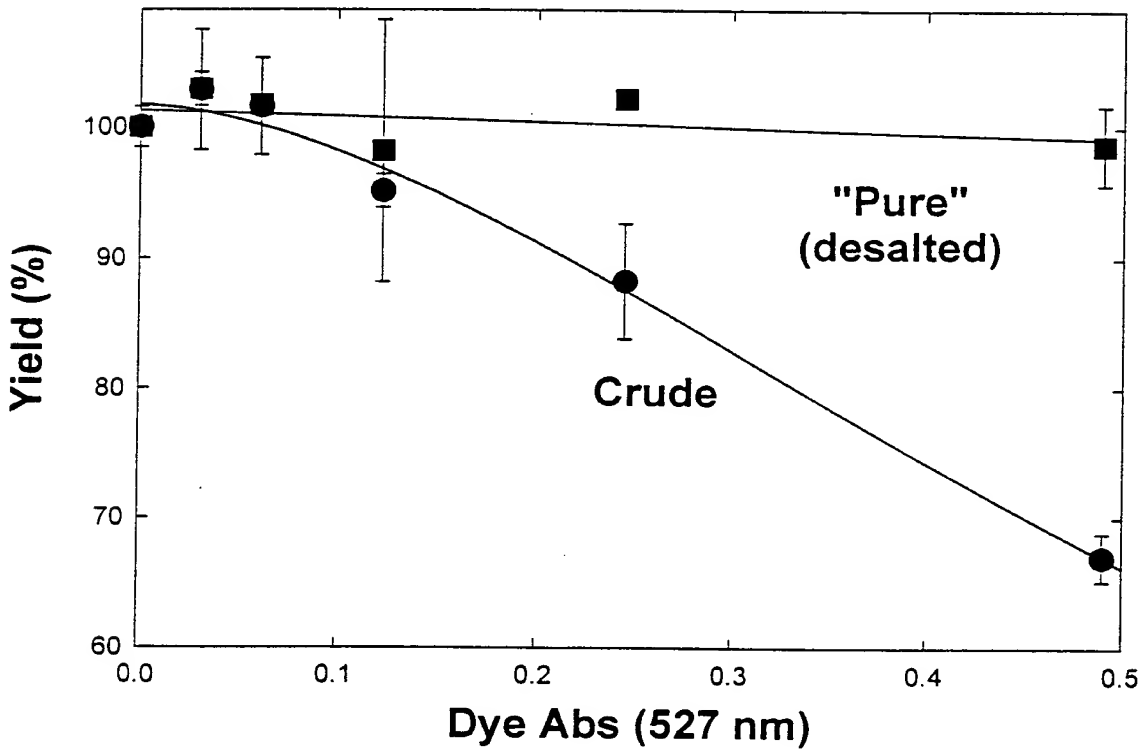

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Figure 12a. PCR yields of Mg, Na and NH_4 purified acid red 1 and acid violet 5 dyes. Na purified as in Figure 11. NH_4 prepared by a. HCl pptn, b. NH_4OH solvation c.. Evaporation of excess NH_4OH. Mg dyes prepared by precipitation using $MgCl_2$ (Mg salts much less soluble than Na salts). Target and PCR conditions were as described in Figure 10.

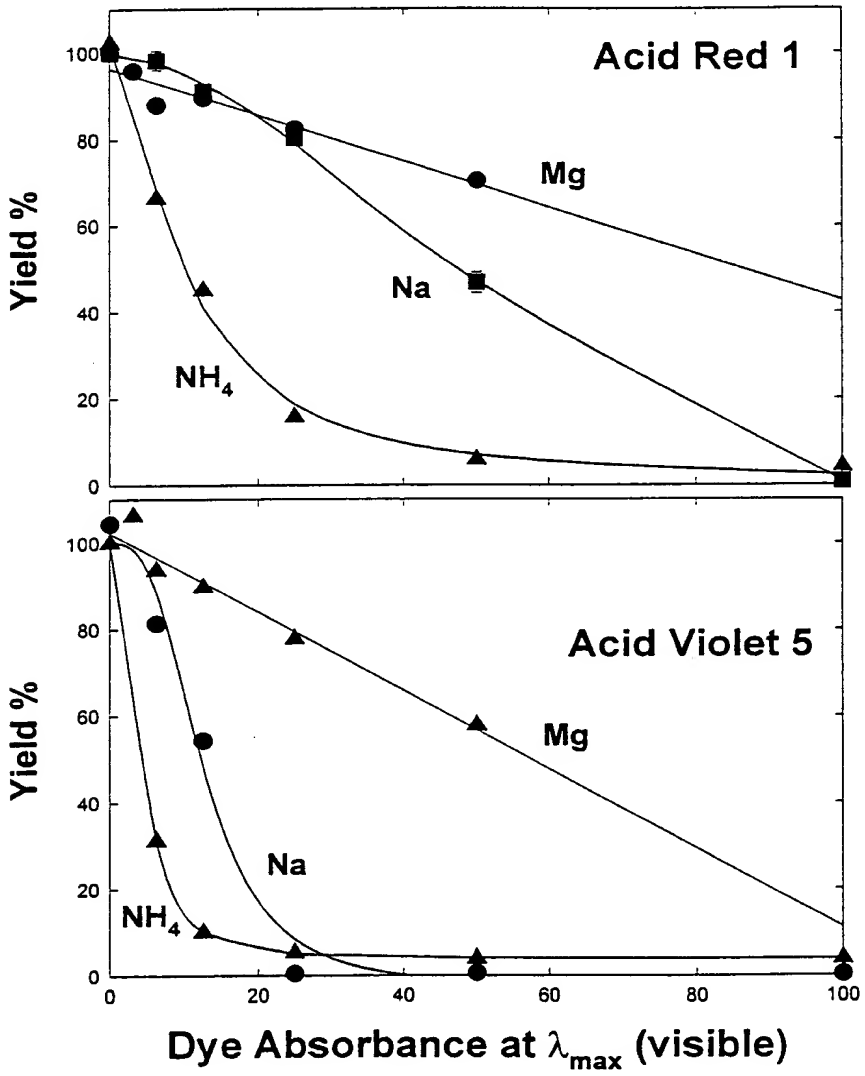

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Figure 12b. PCR yields of Mg, Ca and Zn purified acid red 1. Divalent dyes were prepared as for Mg in Figure 12a. Target and PCR conditions were as described in Figure 10.

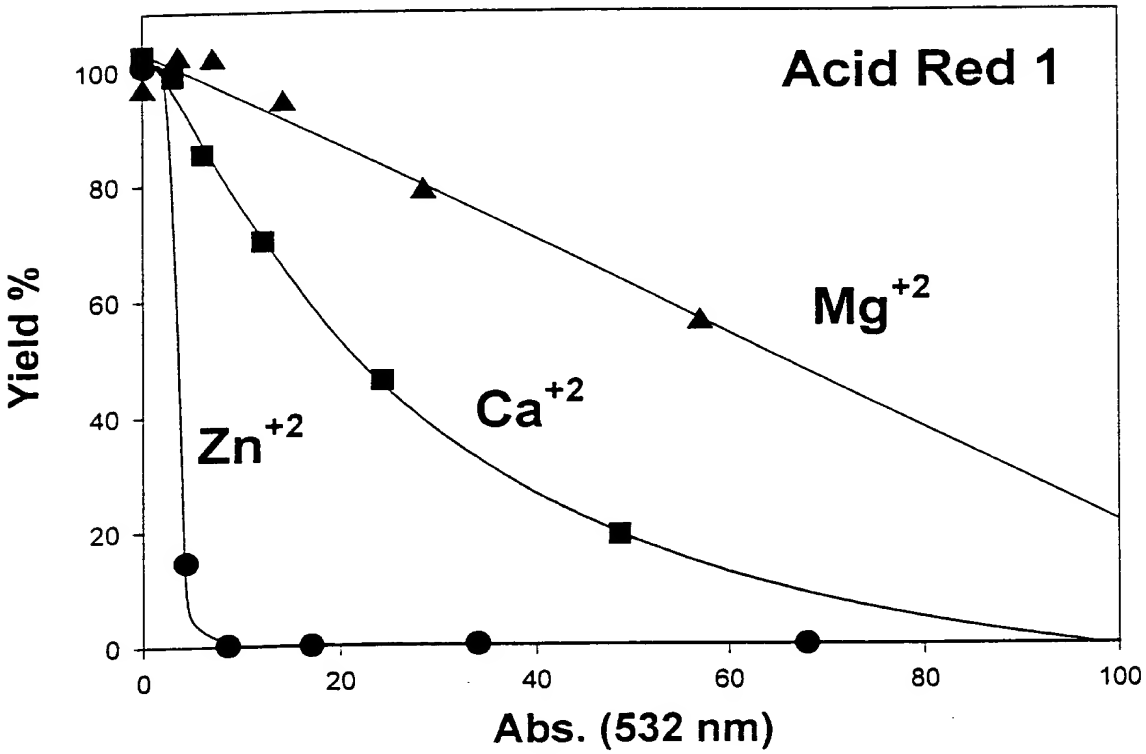

Figure 13. Apparent $[Mg^{+2}]$ of red Taq (80% acid red 1, 20% acid violet 5). $[Mg^{+2}]_{app}$ =0.37+/-0.04mM. Target and PCR conditions were as described in Figure 10. 500, 1500 and 3000mers were different lambda DNA target sizes.

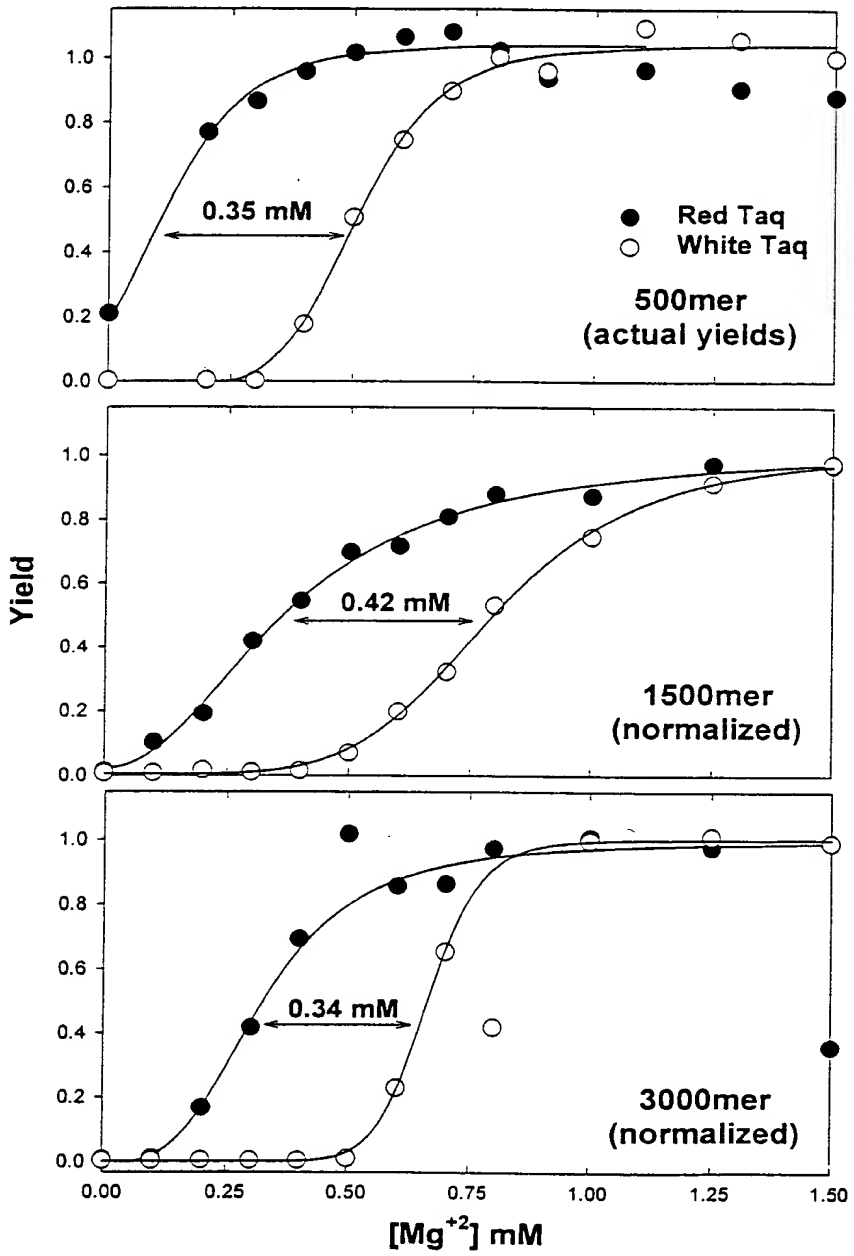

Figure 14. 1% agarose gel contrasting Taq DNA polymerase PCR products formulated without (lanes 2-6) and with (lanes 7-11) optimized analysis reagents. Lane 1,12 are Lambda *Hin*dIII markers. Lanes 2-6 and 7-11 sequentially correspond to amplification targets of length 1,2,3,7 and 10 kb (template=lambda). Absence of product in lane 4 is an artifact.

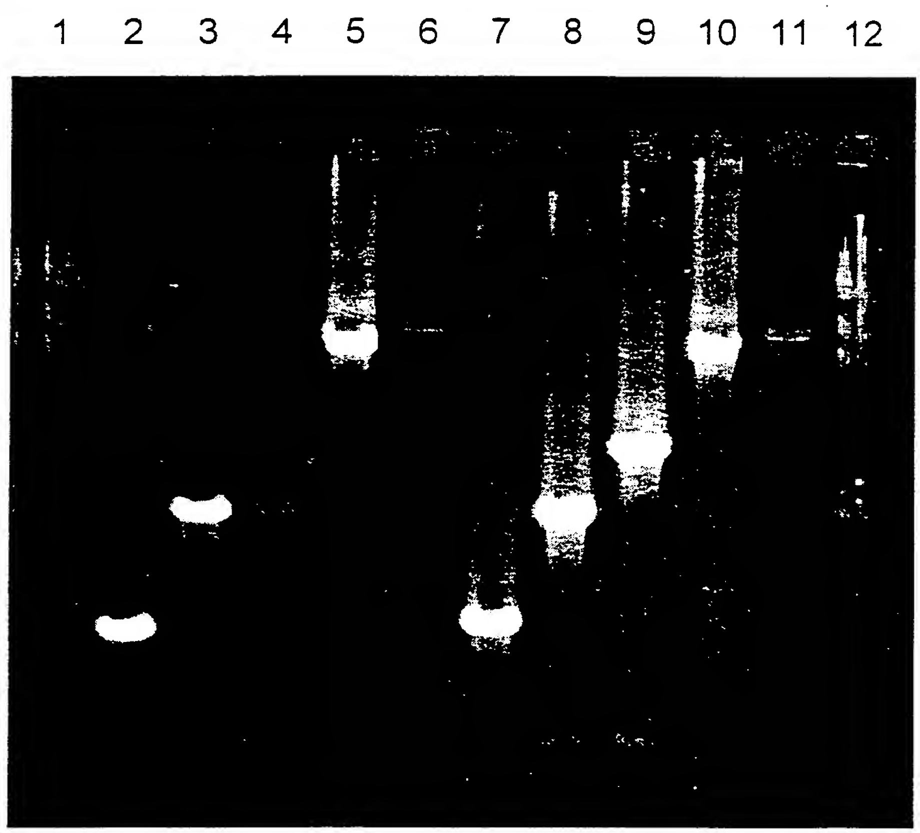

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Figure 15. PCR product yields using red and white Taqs with their respective buffers as a function of product length. At each length white Taq was considered 100%, red Taq was relative to white.

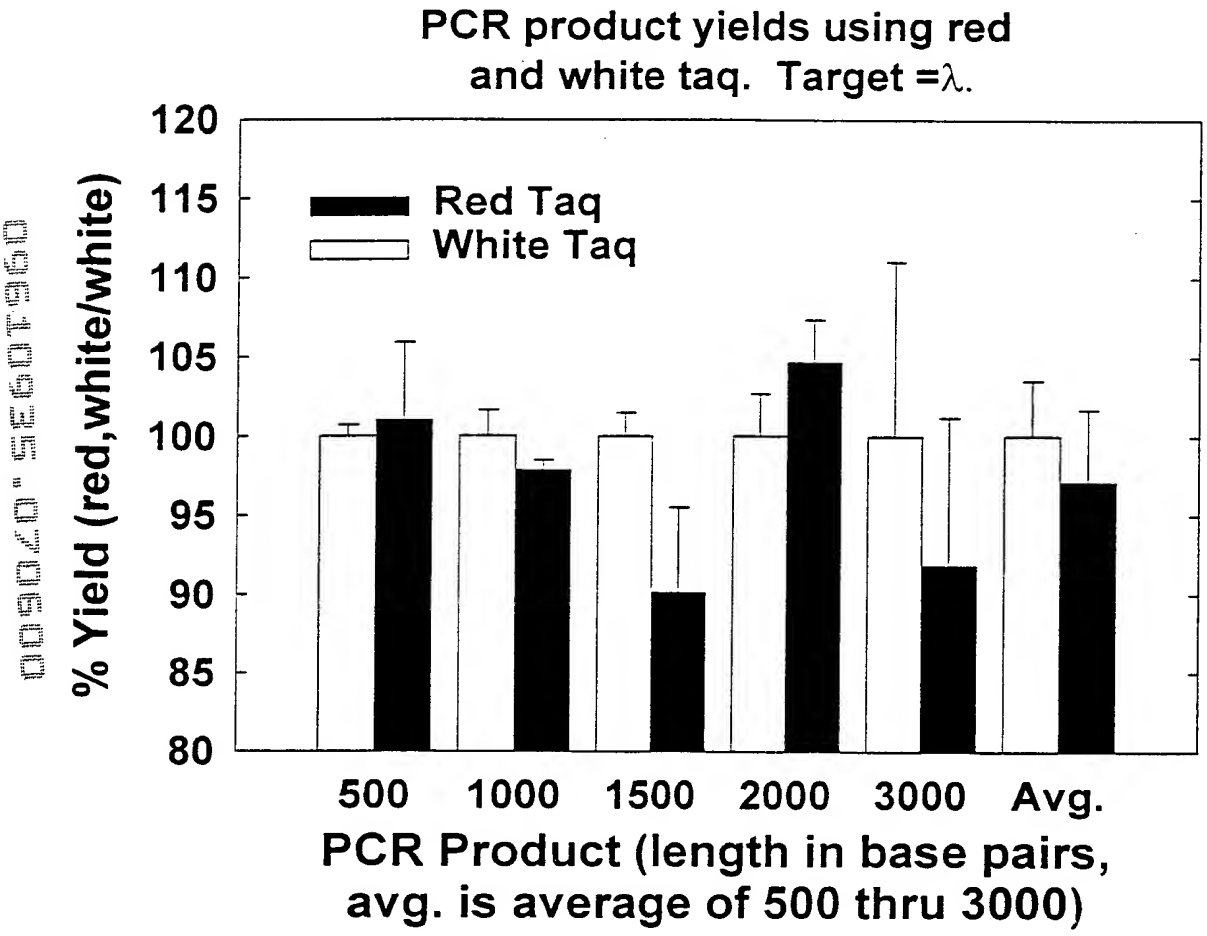

Figure 16. PCR product yields using Taq and Taq + Rediload (Research Genetics). PCR as in Figure 10.

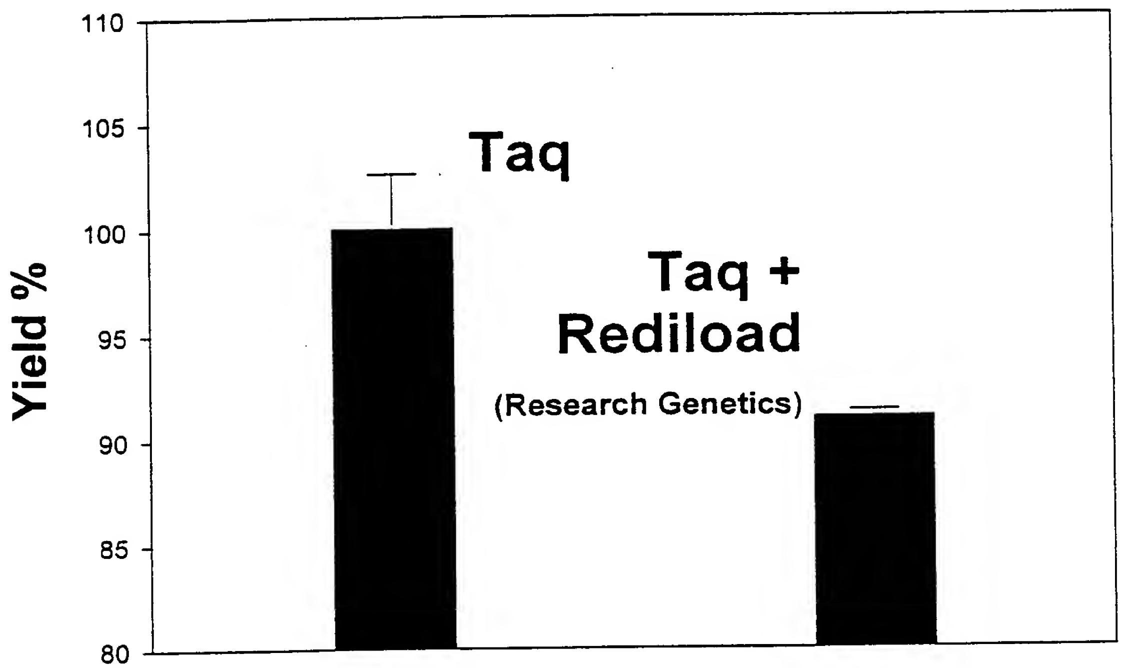

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Figure 17. 4% agarose gel electrophoresis of restriction enzyme digestion of NdeI cut pUC19 in presence (sufficient for use as a tracking dye) and absence of crude Amaranth. Column 1 is a 100 to 1000 base pair (100 bp/band) DNA molecular weight ladder. Columns 2-4 are with dye and decreasing amounts of restriction enzyme (I.e. 5X dilution per column), columns 5-7 are as 2-6 except in absence of dye. Rows are labeled with the appropriate restriction enzyme.